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ORIGINAL ARTICLE

Expression and Prognostic Significance of CD151, c-Met, and Integrin alpha3/alpha6 in Pancreatic Ductal Adenocarcinoma

Guang-Hui Zhu · Chen Huang · Zheng-Jun Qiu · Jun Liu · Zhi-Hua Zhang · Ning Zhao · Zheng-Zhong Feng · Xiu-Hong Lv

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Abstract
Background CD151, c-Met, and integrin alpha3/alpha6 are all involved in the hepatocyte growth factor (HGF)/c-Met signal pathway, which plays an important role in the malignant progression of tumors.
Aims The purpose of this study was to explore the expression and prognostic significance of these proteins in pancreatic ductal adenocarcinoma (PDAC).
Methods We used immunohistochemical methods to investigate the expression patterns of CD151, c-Met, and integrin alpha3/alpha6proteins in 71 patients with PDAC and in ten samples of normal pancreatic tissue. We also assessed correlations between these proteins and clinicopathological parameters and survival of PDAC patients using various statistical methods.
Results CD151, c-Met, and integrin alpha3/alpha6 were all overexpressed in PDAC. CD151 and c-Met overexpressions were significantly associated with TNM stage ($p = 0.001$ and $p = 0.038$, respectively) and lymph node invasion ($p = 0.000$, $p = 0.012$, respectively). A significant positive linear correlation was found between CD151 and c-Met ($r = 0.583$; $p = 0.000$), integrin alpha3 ($r = 0.457$; $p = 0.000$), and integrin alpha6 ($r = 0.671$; $p = 0.000$). Overexpression of CD151, c-Met, integrin alpha3, or integrin alpha6 was related to poor survival of PDAC patients ($p = 0.000$, $p = 0.000$, $p = 0.005$, and $p = 0.003$, respectively), and CD151 and c-Met were independent factors in prognosis of PDAC.
Conclusions CD151, c-Met, and integrin alpha3/alpha6 were all overexpressed in PDAC. CD151 and c-Met might be new molecular markers to predict the prognosis of PDAC patients.

Keywords CD151 · c-Met · Integrin alpha3 · Integrin alpha6 · Pancreatic ductal adenocarcinoma · Prognosis

Guang-Hui Zhu and Chen Huang contributed equally to this work.

G.-H. Zhu · C. Huang · Z.-J. Qiu (✉) · J. Liu · Z.-H. Zhang · N. Zhao
Department of General Surgery, Affiliated First People's Hospital, Shanghai Jiao Tong University, 200080 Shanghai, China
e-mail: qiuwryb@gmail.com

G.-H. Zhu
e-mail: zhuzhu19820515@126.com

C. Huang
e-mail: richard-hc@hotmail.com

J. Liu
e-mail: liujum@yahoo.com.cn

Z.-H. Zhang
e-mail: zhihua-049801@163.com

N. Zhao
e-mail: znbar@126.com

Z.-Z. Feng · X.-H. Lv
Department of Clinical Pathology, Affiliated First People's Hospital, Shanghai Jiao Tong University, 200080 Shanghai, China
e-mail: fzz1976@sina.com

X.-H. Lv
e-mail: lvxiuhong1999@yahoo.com.cn

G.-H. Zhu · C. Huang · Z.-J. Qiu · J. Liu
Department of Shanghai Key Laboratory of Pancreatic Diseases, Shanghai Jiao Tong University, 200080 Shanghai, China

Introduction

Pancreatic cancer is the fourth leading cause of cancer-related mortalities [1]. Patients with pancreatic ductal adenocarcinoma (PDAC), which represents 80–90% of pancreatic cancer cases, have a very poor prognosis: Less than 5% of patients survive 5 years after the diagnosis [2]. This is partly because PDAC is difficult to diagnose in its early stages, and over 85% of tumors exhibit massive metastatic spread at the time of diagnosis [3]. Cancer cells may spread by direct extension from the pancreas to adjacent structures and regional lymph nodes, and the liver, lung, and peritoneum are the most common sites of distant metastasis. The mechanisms underlying the extraordinarily high invasive capacity of PDAC remain elusive. However, some researchers have suggested that altered expression of cell surface membrane molecules, particularly tetraspanins, might be an important factor involved in cancer metastasis [4].

The tetraspanins comprise a large superfamily of cell surface membrane proteins with four transmembrane domains. Tetraspanins are involved in a multitude of biological processes, such as cell proliferation [5], adhesion [6], fusion [7], motility [8], and tumor metastasis [4]. CD151 is the first member of the tetraspanin family to be identified as a promoter of human tumor metastasis [9]. CD151 has been associated with enhanced metastasis of colon [10], prostate [11], lung [12], and liver cancer [13]. Furthermore, a study of pancreatic cancer cell lines linked high CD151 expression with cell motility, which is important to the metastatic process [14]. Tetraspanins are known to form protein complexes with integrins and modulate the adhesion, invasion, and migration capacity of cancer cells in an integrin-dependent manner [15]. These functions can partly explain why tetraspanins might regulate metastasis. The most common complexes are formed between CD151 and integrin alpha3/alpha6 [16, 17], and alpha3 and alpha6 reportedly are overexpressed in human pancreatic tumor cell lines [18]. Sawai et al. [19] reported that high integrin alpha6 expression in PDAC tumor tissue was associated with advanced TNM stage, presence of liver metastases, and poor prognosis. Integrin alpha6 upregulation in patients with PDAC was found to be related to the presence of liver metastasis, lymph node metastasis, retroperitoneal invasion, and poor prognosis [20]. Gesierich et al. [14] reported that pancreatic cancer cell lines and tissues mostly expressed integrin alpha3 at an intermediate level and integrin alpha6 at a high level. In the nude mouse, high integrin alpha6 expression was associated with metastasis [21]. However, another report indicated that pancreatic adenocarcinoma tissues displayed weak integrin alpha6 expression and high integrin alpha3 levels [22], and yet another study revealed a diffuse distribution of integrin alpha 6 on the cell surface and a heterogeneous expression of integrin alpha3 in pancreatic carcinoma tissues [23].

Recent studies have demonstrated that CD151 forms a structural and functional complex with c-Met and integrin alpha3/alpha6 and that it plays an important role in regulating hepatocyte growth factor (HGF)/c-Met signaling in human salivary gland cancer cells and breast cancer cells [24, 25]. The proto-oncogene c-Met encodes a member of the family of receptor tyrosine kinases. The c-Met receptor binds to and is activated by HGF, leading to increased proliferation, augmented motility, and enhanced invasion [26]. Clinical studies have shown that c-Met is overexpressed in many types of human cancer, including breast cancer [27], gastric cancer [28], hepatocellular carcinoma [13], and pancreatic carcinoma [29]. However, the prognostic significance of CD151, c-Met, and integrin alpha3/alpha6 in PDAC remain uncertain.

The goals of this study were to further explore the expression patterns of CD151, c-Met, and integrin alpha3/alpha6 in patients with PDAC and to determine whether these proteins could be prognostic factors in PDAC. We investigated the expression of these proteins in 71 patients with PDAC and in ten samples of normal pancreatic tissue using immunohistochemical methods. Furthermore, correlations between these proteins and clinicopathological parameters and survival of PDAC patients were assessed.

Materials and Methods

Patient Selection

Seventy-one patients with PDAC who underwent radical surgery at our hospital between August 1993 and January 2009 were included in this study. There were no particular selection criteria for the cases. This study was approved by the ethical committee of the First Affiliated Hospital of Shanghai Jiao Tong University. Written consent was acquired from relatives of all patients enrolled in the study. The mean age of the patients was 64 years (range: 40–80). None of the patients had received preoperative radiation or chemotherapy. Experienced pathologists provided detailed pathological diagnosis of the excised tumors according to the seventh edition of the tumor-node-metastasis (TNM) classification of the International Union Against Cancer [30]. Histological tumor grade was determined following the World Health Organization's classification system [31]. Table 1 summarizes the clinicopathological characteristics of the study population. Ten samples of normal pancreatic tissue were obtained from donors (who gave informed

Table 1 Summary of clinical and pathological characteristics of patients with PDAC

Characteristics	*n*
Age (years)	
Mean	64.15 ± 9.91
Range	40–80
Gender	
Male	50
Female	21
Tumor location	
Head	62
Body and tail	9
Tumor size (cm)	
≤2	10
>2	61
TNM stage	
Ia	2
Ib	16
IIa	25
IIb	23
III	0
IV	5
Lymph node status	
Negative	43
Positive	28
Histopathological grading	
Grade 1	14
Grade 2	45
Grade 3	10
Not classified	2

consent) via regular multi-organ donor procedures. Each patient received a CT scan twice a year to detect the recurrence of cancer. Follow-ups were continued until the death of the patient. Patient survival data were obtained by telephone contact and direct home visit. The follow-up period ended on March 14, 2010.

Immunohistochemical Procedures and Evaluation

Pancreatic cancer specimens and normal pancreatic tissues were fixed in 4% formalin and embedded in paraffin. The paraffin-embedded samples were serially sectioned at 4 μm thickness, mounted on slides treated with poly-L-Lysine, and used for immunohistochemistry. Tissue sections (4 μm) were deparaffinized in xylene and rehydrated through a graded ethanol series. Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide in methanol for 10 min. Antigen retrieval was achieved by microwaving the sections in 0.01 M citrate buffer (pH 6.0) for 10 min followed by cooling for 30 min. Slides were washed in PBS and incubated in 10% normal goat serum for 10 min to block nonspecific binding of the antibodies. Sections were then incubated with mouse anti-human CD151 monoclonal antibody (sc-80715, 1:100 dilution, Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit anti-human c-Met monoclonal antibody (ab51067, 1:100 dilution, abcam, Cambridge, UK), mouse anti-human integrin alpha3 monoclonal antibody (sc-13545, 1:100 dilution, Santa Cruz Biotechnology), and rabbit anti-human integrin alpha6 polyclonal antibody (sc-10730, 1:100 dilution, Santa Cruz Biotechnology) at room temperature for 60 min. Binding of antibodies was visualized via a two-step immunohistochemical procedure using UltraSensitive™ S-P Universal (Anti-Mouse/Rabbit) Detection Reagent (HRP) (KIT-9730, Fuzhou Maixin Biotech Inc., Fujian, China) according to the manufacturer's instructions. The reaction products were visualized using DAB as a chromogen followed by nuclear counterstaining with hematoxylin. The negative controls were prepared by substituting PBS for the primary antibodies. Results were evaluated independently by two pathologists without prior knowledge of the patients' clinical data. We determined the total score by estimating the percentage of cells that stained for CD151, c-Met, and integrin alpha3/alpha6 and by weighting based on assessment of the intensity of the stain, as previously described [10, 13, 45]. The percentage of positive cells was rated as follows: 1 point, up to 10% positive cells; 2 points, 11–50% positive cells; 3 points, 51–70% positive cells; 4 points, >71% positive cells. The staining intensity was classified as 0, 1, 2, or 3 points for no staining, weak, moderate, and strong intensity, respectively. Specimens were divided into four groups according to the sum of the two score: −, 1–2 points; +, 3 points; ++, 4–5 points, and +++, 6–7 points; 3–7 points were considered as positive for a tumor specimen. For statistical reasons, tumors were classified into the following two groups: (−) and (+), low reactivity group; (++) and (+++), high reactivity group.

Statistical Analysis

SPSS for Windows (Version 13.0; Chicago, IL, USA) was used to conduct statistical analyses. Categorical variables were assessed using the Chi-square test and Fischer's exact test (two-sided). Spearman's rank correlation coefficient test was used to test for associations between ordinal variables. Univariate analyses of overall survival and survival curves were performed following the Kaplan–Meier method, and the Cox proportional hazards model was used for multivariate analysis. All factors that were significant for predicting overall survival by univariate analysis were

included in the multivariate Cox regression analysis. $p < 0.05$ was considered to be statistically significant.

Results

Expression of CD151, c-Met, and Integrin alpha3/alpha6 Proteins

In the PDAC samples analyzed in this study, CD151, c-Met, integrin alpha3, and integrin alpha6 immunoreactivities all displayed intense diffuse cytoplasmic and membrane staining in tumor cells (Fig. 1a–d). In contrast, expression of these proteins was not detected in the ten normal pancreatic samples. CD151, c-Met, integrin alpha3, and integrin alpha6-positive specimens were detected in 58 (81.69%), 49 (69.01%), 49 (69.01%), and 60 (84.51%) of the 71 patients, respectively. Negative (−), weak (+), moderate (++), and strong (+++) expression staining categories were detected in 13, 23, 29, and six cases for CD151; 22, 6, 28, and 15 cases for c-Met; 22, 10, 35, and four cases for integrin alpha3; and 11, 14, 30, and 16 cases for integrin alpha6, respectively.

Relationship Between CD151 and Other Associated Proteins

Spearman rank correlation revealed a significant positive correlation between CD151 and c-Met ($r = 0.583$; $p = 0.000$), integrin alpha3 ($r = 0.457$; $p = 0.000$), and integrin alpha6 ($r = 0.671$; $p = 0.000$) (Table 2).

Correlations Between CD151, c-Met, and Integrin alpha3/alpha6 Expression and Various Clinicopathologic Factors

To obtain a better understanding of the clinical significance of CD151, c-Met, integrin alpha3, and integrin alpha6 expression in PDAC, we examined the correlation between the expression of these proteins and a series of clinicopathological characteristics. As shown in Table 3, CD151 overexpression was significantly associated with TNM stage ($p = 0.001$) and lymph node invasion ($p = 0.000$). However, other clinical characteristics were not directly related to the expression of CD151. c-Met overexpression was significantly correlated with TNM stage ($p = 0.038$) and lymph node invasion ($p = 0.012$) but not with the other clinicopathological variables. None of the clinical

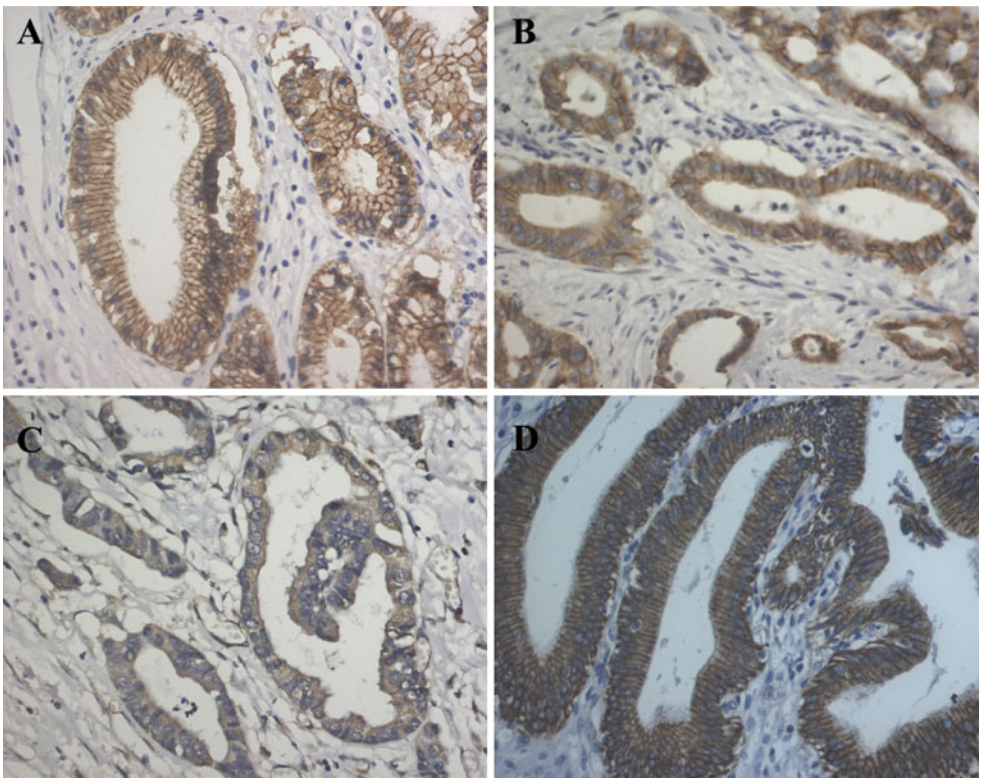

Fig. 1 Expression of CD151, c-Met, and integrin alpha3/alpha6 proteins in pancreatic cancer tissues determined using immunohistochemical methods. **a** CD151, **b** c-Met, **c** integrin alpha3, and **d** integrin alpha6 in PDAC demonstrated strong membrane and cytoplasmic staining, which was scored as overexpression (**a–d**, original magnification ×400)

Table 2 Relationship between CD151 and other investigated proteins in PDAC

Other protein expression	CD151 expression				Spearman rank correlation	
	(−) ($n = 13$)	(+) ($n = 23$)	(++) ($n = 29$)	(+++) ($n = 6$)	r	p value
c-Met						
Negative ($n = 22$)	13	6	2	1		
Weak ($n = 6$)	0	1	5	0		
Moderate ($n = 28$)	0	14	12	2		
Strong ($n = 15$)	0	2	10	3	0.583	0.000
Integrin alpha3						
Negative ($n = 22$)	9	11	2	0		
Weak ($n = 10$)	0	2	8	0		
Moderate ($n = 35$)	4	8	18	5		
Strong ($n = 4$)	0	2	1	1	0.457	0.000
Integrin alpha6						
Negative ($n = 11$)	10	0	1	0		
Weak ($n = 14$)	3	6	5	0		
Moderate ($n = 30$)	0	17	10	3		
Strong ($n = 16$)	0	0	13	3	0.671	0.000

Table 3 Comparison of CD151, c-Met, and integrin alpha3/alpha6 immunohistochemistry with clinicopathological features in patients with PDAC

Parameters	n	CD151			c-Met			Integrin alpha3			Integrin alpha6		
		Low	High	p	Low	High	p	Low	High	p	Low	High	p
Age (years)													
≤60	26	14	12		12	14		14	12		9	17	
>60	45	21	24	0.560[a]	16	29	0.379[a]	18	27	0.259[a]	16	29	0.936[a]
Gender													
Male	50	23	27		19	31		24	26		16	34	
Female	21	12	9	0.391[a]	9	12	0.702[a]	9	12	0.808[a]	9	12	0.382[a]
Tumor location													
Head	62	31	31		25	37		30	32		22	40	
Body and tail	9	4	5	1.000[b]	3	6	1.000[b]	2	7	0.171[b]	3	6	1.000[b]
Tumor size (cm)													
≤2	10	5	5		3	7		5	5		4	6	
>2	61	30	31	1.000[b]	25	36	0.730[b]	27	34	0.746[b]	21	40	0.733[b]
TNM stage													
I	18	13	5		10	8		7	11		8	10	
IIa	25	16	9		12	13		15	10		11	14	
IIb, III, IV	28	6	22	0.001[a]	6	22	0.038[a]	10	18	0.172[a]	6	22	0.146[a]
Lymph node status													
Negative	43	29	14		22	21		22	21		19	24	
Positive	28	6	22	0.000[a]	6	22	0.012[a]	10	18	0.201[a]	6	22	0.050[a]
Grade													
G1 + G2	59	31	28		23	36		28	31		20	39	
G3	10	2	8	0.087[b]	3	7	0.732[b]	3	7	0.494[b]	3	7	1.000[b]

[a] χ^2 test

[b] Fisher's exact test

Table 4 Relationship between CD151and c-Met expression and recurrence of PDAC

	CD151		*p*	c-Met		*p*
	Low	High		Low	High	
No recurrence	7	2	0.277[a]	7	2	0.029[a]
Recurrence	23	19		15	27	

[a] Fisher's exact test

characteristics were directly related to the expression integrin alpha3 and integrin alpha6.

Correlations Between CD151 and c-Met Expression and Tumor Recurrence

Seventy-one patients with PDAC who underwent radical surgery were included in this study. Sixty-six patients (92.96%) at stage I and II underwent radical surgery with the aim of achieving a curative resection. Of these 66 patients, we lost track of six during the observation period and 42 experienced recurrence of pancreatic cancer. Additionally, nine patients died without recurrence of pancreatic cancer. As described in Table 4, where curative resection was achieved, c-met level but not CD151 were correlated with future recurrence rates.

Survival Analysis and the Prognostic Value of CD151, c-Met, and Integrin alpha3/alpha6 Expression

Overall survival time was defined as the period from the day of surgery to the death of the patient. Death from a cause other than cancer relapse or survival at the end of observation period was considered to be a censoring event. We lost track of six patients during the observation period, so follow-up data were available for 65 of the original 71 patients. The median follow-up time was 14.0 months. The survival rate was 55.4 and 29.2% at 1 and 2 years. Forty-two patients had died of pancreatic cancer recurrence at the endpoint of the study, and three patients were still alive 5 years after their operation. Survival analysis based on single protein expression demonstrated that overexpression of CD151, c-Met, integrin alpha3, and integrin alpha6 was associated with shorter survival ($p = 0.000$, $p = 0.000$, $p = 0.005$, and $p = 0.003$, respectively, Fig. 2). All factors that were significant for predicting overall survival in the

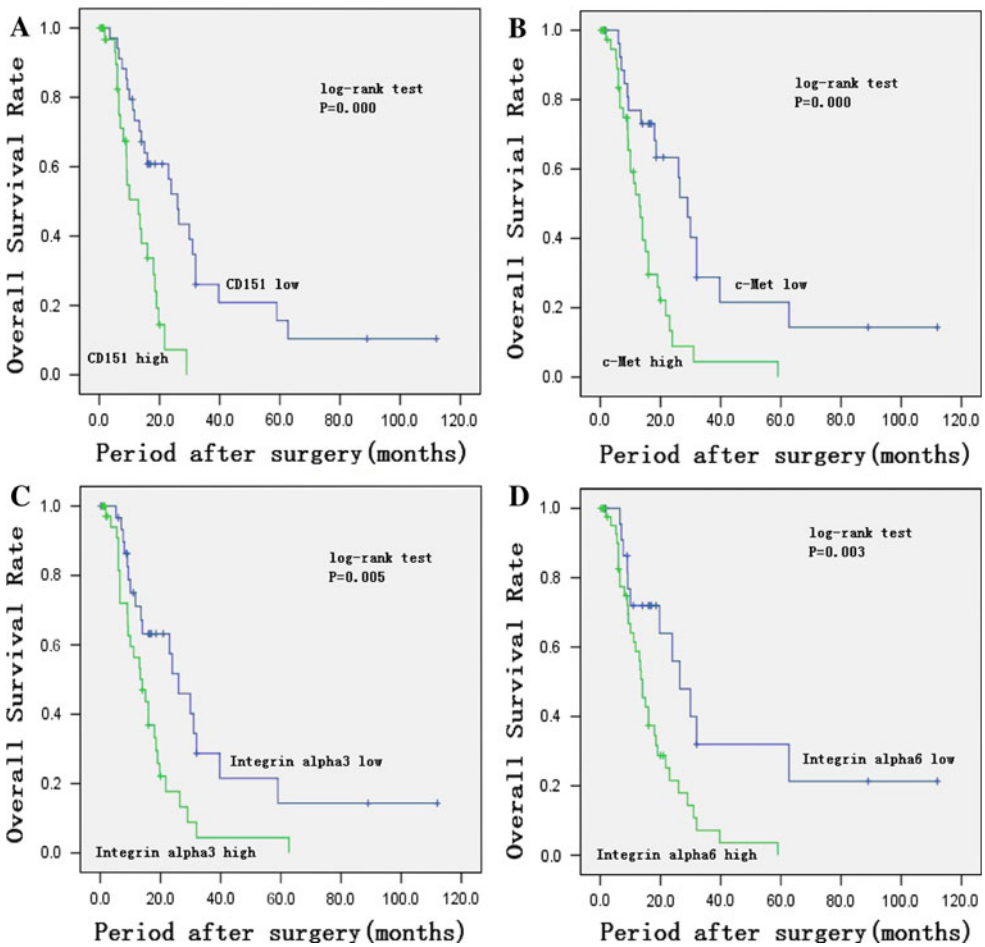

Fig. 2 Survival curves of PDAC patients relative to **a** CD151, **b** c-Met, **c** integrin alpha3, and **d** integrin alpha6 expression. **a** PDAC patients with low CD151 expression survived longer than those with high CD151 expression ($p = 0.000$). **b** High expression of c-Met indicated a shorter overall survival for patients with PDAC ($p = 0.000$). **c** The overall survival rate of PDAC patients with high integrin alpha3 expression was significantly lower than that of patients with low integrin alpha3 expression ($p = 0.005$). **d** PDAC patients with low integrin alpha6 expression survived longer than those with high integrin alpha6 expression ($p = 0.003$)

Table 5 Univariate and multivariate analysis of overall survival of 71 patients with PDAC

Variables	Univariate analysis	Multivariate analysis		
	p	*p*	Relative risk	95% confidence cinterval
Age (≤60 years vs. >60 years)	0.696			
Gender (male vs. female)	0.341			
Location(head vs. body + tail)	0.316			
Size (≤2 cm vs. >2 cm)	0.257			
Histologic grade (G1 + G2 vs. G3)	0.107			
Lymph node status (negative vs. positive)	0.062			
TNM stage(Ia/Ib/IIa/IIb/III/IV)	0.067			
CD151 (high vs. low)	0.000*	0.006*	2.605	1.308–5.187
c-Met (high vs. low)	0.000*	0.010*	2.427	1.239–4.754
Integrin alpha3 (high vs. low)	0.751	0.212		
Integrin alpha6 (high vs. low)	0.032*	0.245		

* Significant at $p < 0.05$ level

univariate analysis were included in the multivariate Cox regression analysis, which revealed that CD151 and c-Met expressions could be independent prognostic factors for PDAC patients ($p = 0.006$ and $p = 0.010$, respectively, Table 5), but this was not true for integrin alpha3 and integrin alpha6 (Table 5).

Discussion

In this study, we confirmed that expression of CD151 was markedly increased in PDAC tissues compared with that in normal pancreas tissues. This result is consistent with that of previous reports [18, 20]. We also identified a relationship between high CD151 expression and poor prognosis of PDAC patients. This finding is supported by the fact that aggressive clinicopathological characteristics, such as lymph node invasion and low histological grade of PDAC, were significantly more frequent in patients with high CD151 expression than in those with low expression. Although previous studies have shown that overexpression of CD151 predicts poor clinical outcome in patients with hepatocellular carcinoma [13] and prostate cancer [11], to the best of our knowledge ours is the first report to show that CD151 was an independent factor in prognosis of PDAC. CD151 is a membrane protein of the tetraspanin superfamily; members of this family have four transmembrane domains, two extracellular loops, and intracellular NH2- and COOH-terminal domains [32]. These features enable CD151 to act as an adaptor or organizer by assembling multimolecular complexes of cell surface proteins. Hence, CD151 is involved in numerous biological processes, including cell proliferation [7], adhesion [33], motility [34], angiogenesis [35], formation of hemidesmosomes [16], and metastasis [9]. Zijlstra et al. [36] showed that a CD151 antibody prevented tumor cell detachment from the primary tumor site. Moreover, monoclonal antibodies to CD151 inhibited in vivo metastasis of human cancer cells, and transfection of CD151 cDNA into different tumor cell lines resulted in enhanced cell motility and metastasis [9]. The expression of CD151 has been evaluated in various cancers [10–13, 20, 37, 38]. CD151-positive patients tended to have lower survival rates [10–13]. Our data support this finding and suggest that CD151 plays an important role in cancer biology.

Like other tetraspanins, CD151 forms complexes by interacting with a variety of transmembrane and cytosolic proteins that are required for its function [14]. The most prominent partners are integrins, which are thought to regulate in–out signaling. The CD151-integrin alpha3/alpha6 association has been shown to be highly specific and stable [15, 16, 39], and CD151 has been shown to potentiate ligand binding activity of integrin alpha3beta1 and alpha6beta1 [40, 41]. CD151 cooperation with integrins plays an important role in the biological behavior of tumors, especially metastasis [4]. Metastasis involves a complex and multistep cascade of processes, during which tumor cells detach from matrix and neighboring cells, migrate through the surrounding stroma, enter the circulatory system, and finally arrest and undergo extravasation and growth at a secondary site [42]. CD151 recruits integrin alpha3beta1 to form cell–cell contacts through a multimolecular complex that includes PKC, PTPμ, E-cadherin, and β-catenin. This phenomenon can partly explain how CD151 regulates integrin-dependent cadherin-mediated adhesion [43]. CD151 also regulates cell adhesion dependent of integrin-associated PKC and CDC42 signaling [44]. In addition, CD151 plays a key role in selectively

strengthening alpha6beta1 integrin-mediated adhesion to laminin-1 [41]. Two different adhesion complexes (CD151-integrin alpha3beta1 and CD151-integrin alpha6-beta1) have been identified in prostate cancer [45]. CD151-integrin alpha3beta1/alpha6beta1 complexes enhance c-Jun activity through the activation of FAK, Src, and MAPK [8].

CD151 has been shown to be involved in regulating HGF/c-Met signaling in some cancer cells [24, 25]. HGF is a pleiotropic polypeptide growth factor that regulates cell proliferation, migration, and invasion [46]. These diverse biological effects are mediated through its receptor, c-Met. In several studies, knockdown of CD151 or integrin a3/a6 expression almost completely abrogated HGF-promoted cell migration [24, 47]. In contrast, forced expression of CD151 resulted in an increase in HGF-dependent biological effects [24]. Herein, we provide clinical evidence demonstrating that alterations in expression of c-Met and integrin alpha3/alpha6 occur in patients with PDAC. We confirmed that elevated CD151 expression was significantly correlated with intense c-Met, integrin alpha3, and integrin alpha6 expressions, which suggests that CD151 may form a complex with these proteins. Moreover, we demonstrated that c-Met overexpression was significantly correlated with TNM stage and lymph node invasion. Our findings support the involvement of the complex in the malignant biological behavior of PDAC. Moreover, where curative resection was achieved, c-met level were correlated with future recurrence rates. To further explore the prognosis value of the complex, we included CD151, c-Met, integrin alpha3, and alpha6 expression in the multivariate Cox regression analysis. Interestingly, patients with CD151 or c-Met overexpression had significantly shorter survival. Thus, CD151 and c-Met are independent prognostic factors for unfavorable prognosis.

In the present study, we provide clinical evidence that supports the upregulation of HGF/c-Met signaling in PDAC. Our data support the premise that CD151, c-Met, and integrin alpha3/alpha6 are key components of a complex that regulates this signaling pathway. Moreover, our results show that CD151 and c-Met are potential predictors of poor prognosis. Therefore, a routine immunohistochemical evaluation of these proteins may be helpful for the clinical assessment of tumor behavior and prognosis in patients with PDAC. Further studies are needed to establish the biological significance of CD151 and c-Met expression and to examine the possibility of using them as therapeutic targets in treatment of PDAC.

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